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Retroviral Gene Transfer into the Intestinal Epithelium

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ABSTRACT

The epithelial cells of the gastrointestinal tract may be attractive targets for somatic gene therapy. In these studies, we have used rats and mice to explore the feasibility of gene transfer into the small intestinal epithelium using retroviral vectors. The first series of experiments was conducted in mature Sprague-Dawley rats using an ecotropic retroviral vector that has bacterial β-galactosidase (β-Gal) as the reporter gene. The vector was introduced into the lumen of ligated segments of terminal ileum. After a 4-hr exposure period, the ligatures were removed. Sham-operated animals were subjected to the same ligation procedure but received only tissue culture medium in the ligated segment. All animals were sacrificed 6 days later, and tissue from both the experimental segment and an upstream control segment was assessed for cytoplasmic β-Gal activity using X-Gal histochemistry. Expression of the reporter gene was observed in the crypt epithelium of tissue exposed to the vector. In the villus epithelium, high background staining precluded accurate assessment of reporter gene expression. To obviate the latter problem, we sought an alternative reporter gene for which there would be no background staining in control animals. We repeated the experiments with β-glucuronidase as the reporter gene in MPS VII mutant mice, which are devoid of this enzyme. In these studies, ileal segments exposed to the vector demonstrated expression of the reporter gene in both the crypt and villus epithelium 4 days after exposure. These results indicate that genes can be transferred into the intestinal epithelium using retroviral vectors introduced luminally. These studies constitute an encouraging first step in the assessment of the intestinal epithelium as a site for somatic gene therapy.

OVERVIEW SUMMARY

There are a number of congenital disorders of the intestinal epithelium that could be amenable to gene therapy (*e.g.*, cystic fibrosis, transporter deficiencies, *etc.*). In addition, the intestine could be used as an alternative site to correct metabolic disorders, such as phenylketonuria, and secretory disorders, such as hemophilias. This paper presents *in vivo* studies in both a rat model and a mouse model using retroviral vectors delivered into the intestinal lumen. The results show successful gene transfer into epithelial cells and thus pave the way for future experiments designed to improve the efficiency of this process.

INTRODUCTION

THE INTESTINAL EPITHELIUM is a continuously renewing monolayer that occupies the interface between the internal and external milieu. Its role in digestion and absorption is facilitated by its extremely large surface area, which results primarily from the presence of villi. Villus epithelial cells are replaced every 2–3 days with cells emerging from the crypts of Lieberkühn. Each villus is fed by 10 or more crypts (Komuro and Hashimoto, 1990), each of which has a zone of stem cells toward its base (Cheng and Leblond, 1974). Kinetic analysis following labeling with [^{3}H]thymidine has led to the prediction that there are between 4 and 16 stem cells per crypt (Potten and

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Loeffler, 1987). Daughters of the crypt stem cells enter a transit zone where they divide approximately four times before leaving the proliferative cycle (Potten and Loeffler, 1987). They then move onto the villi where they acquire various differentiated functions (Gordon, 1989). The large surface area of the epithelium, together with its ease of access *via* the intestinal lumen, makes it an attractive potential site for somatic gene therapy.

Gene transfer into the intestinal epithelium would have two general applications in the field of gene therapy: (i) the correction of genetic and acquired disorders that affect the epithelium, and (ii) as an alternative site of expression of proteins normally expressed in some other tissue. The intestinal derangements associated with cystic fibrosis (CF) would be a good example for the first application. Three of the four published mouse models for CF (Colledge *et al.*, 1992; Dorin *et al.*, 1992; Snouwaert *et al.*, 1992; O'Neal *et al.*, 1993) have severe pathology of the intestine that leads to intestinal obstruction followed shortly by death. Although other genetic disorders of the intestinal epithelium are relatively rare, the epithelium may be used as an ectopic site for correction of various metabolic disorders and deficiencies of secreted proteins. Inasmuch as liver and intestine share numerous metabolic pathways, Jones *et al.* (1990) have suggested that disorders such as deficiencies in the urea cycle or in phenylketonuria may be amenable to intestinal gene therapy. Likewise, the fact that the intestinal epithelium is capable of secreting a foreign protein into the circulatory system (Sweetser *et al.*, 1988) suggests its use for production of proteins such as specifically engineered antibodies, clotting factors, antiproteases, and protein hormones.

The advantages of the intestine as a site for somatic gene therapy are several fold. First is its ease of access *via* the luminal route which, in humans, would allow direct *in vivo* delivery of vectors using standard endoscopic procedures. A second advantage is that the stem cells, which are the logical targets for gene therapy, are continuously proliferating, thus allowing the deployment of retroviral vectors that are already approved for human investigation. Given that some applications of somatic gene therapy, such as correction of metabolic disorders, may require substantial numbers of cells to be expressing a critical enzyme, a third advantage of the intestinal epithelium is its large mass.

The goal of the present studies was to determine whether gene transfer into the intestinal epithelium could occur *via* luminal delivery of replication-incompetent retroviral vectors in rodent models. Although human application would utilize endoscopic procedures, the lack of availability of such instruments for small rodents necessitated a surgical approach for these exploratory studies. The vectors were introduced into ligated segments of ileum for a 4-hr exposure period. Animals were killed 4–7 days later and assayed for expression of the reporter gene. The results indicate successful gene transfer in both rats and mice, albeit at relatively low efficiency.

MATERIALS AND METHODS

Animals and chemicals

Adult Sprague-Dawley (Crl:CD[SD]BR) male rats weighing 150–175 grams were btained from Charles River Laboratories (Portage, MI). Male MPS VII mice (gus^{mps}/gus^{mps}) from the B6.C-H-2^{bm1}/ByBir-gus^{mps}/ + mutant strain aged 1–5 months (13–29 grams) from The Jackson Laboratory (Bar Harbor, ME) were used. The animals were maintained at 21°C + 1°C with food and water *ad libitum*. All histochemical substrates were from Sigma Chemical (St. Louis, MO).

Retroviral vectors

Zen$^+$β-Gal (courtesy of Dr. Philippe Soriano) is a recombinant derivative of Moloney murine leukemia virus with the bacterial β-galactosidase (β-Gal) gene driven by the viral long terminal repeat (LTR). It was propagated in the ecotropic packaging cell line GP$^+$E86 (Markowitz *et al.*, 1988) and routinely yielded titers in the order of 2–3 × 10^5 cfu/ml. The NTK-βGEO construct has the rat β-glucuronidase gene driven by the herpes simplex thymidine kinase promoter (Wolfe *et al.*, 1990). It was propagated in a clone of GP + E86 and concentrated using the Centricell filtration method to yield titers of 10^7 cfu/ml. In all experiments, Polybrene (8 μg/ml) was added to the vector solutions immediately prior to use.

Surgical protocols

Both rats and mice were subjected to the same surgical procedure. Following isoflurane anesthesia (Anaquest, Madison, WI), an incision was made in the lower abdomen. The first Peyer's patch proximal to the ileocecal junction was identified, and an ileal segment (1.5–3 cm) immediately rostral was gently cleared of its chyme by flushing with phosphate-buffered saline (PBS). The segment was ligated with a coarse thread at both ends, and an appropriate volume (1.5 ml for rats, 0.5 ml for mice) of the respective retroviral vector (experimental) or culture medium (sham) was then introduced *via* a 25-gauge needle. With these volumes, the segments were distended sufficiently to expose the intestinal crypts (Sandberg *et al.*, 1994). The animals were allowed to recover from this initial surgery, then 4 hr later they were reanesthetized and the coarse thread ligatures were removed. Fine silk ligatures were loosely anchored at both ends of the experimental segment for recognition at the time of autopsy.

The rats tolerated the procedure very well and were in good health and gaining weight when sacrificed 6 days post-surgery. In contrast, the MPS VII mice displayed high mortality. With only 9 out of the 29 operated mice surviving on the fourth day post-surgery, these animals were sacrificed at this time rather than waiting for the 6-day period used for the rats. The MPS VII mice have a high mortality rate from their severe degenerative disease (Vogler *et al.*, 1990; Birkenmeier *et al.*, 1991). Of the 9 surviving mice, 4 were sham-treated and 5 were vector-treated.

Histochemical detections

For both rats and mice, at the time of sacrifice the experimental segment (as defined by the marker ligatures) was removed together with a control segment taken 10 cm and 6 cm upstream, respectively. After flushing with 0.9% NaCl to remove luminal contents, the segments were frozen in OCT (Miles, Elkhart, IN) f r subsequent cryostat sectioning and histochemical analyses. Frozen sections (8–10 μm) were made

transverse t the longitudinal axis of the ileum. Consecutive sections were collected at varying intervals along the length of the respective segments to be analyzed. In the rat tissues, β-Gal was detected histochemically using the chromagen X-Gal as a substrate under standard conditions. A section f transgenic mouse liver expressing bacterial β-Gal (courtesy of Dr. Savio Woo) was run with each set as a positive control. In the MPS VII mouse tissues, β-glucuronidase was detected using naphtol-AS-BI β-D-glucuronide and pararosaniline hydrochloride as described by Birkenmeier *et al.* (1989). A section of nonmutant liver expressing β-glucuronidase was run as a positive control. In both rat and mouse studies, the sections were scored blindly, and positive epithelial cells in the villus and crypt regions were counted in both upstream (control) and experimental segments.

RESULTS

β-*Gal expression in rats*

Because endogenous lactase present in the apical membrane of villus epithelial cells is also detected by X-Gal histochemistry, observers scored as positive cells only those which contained cytoplasmic staining (the expected location of the bacterial β-Gal reporter gene). The number of rats exhibiting positive β-Gal scores from this procedure is shown in Table 1A. It can be seen that in the experimental segment, all animals displayed positive cells in the crypt epithelium, and 2 out of 3 animals had positive villus epithelial cells. None of the animals had positive cells in the crypts of the upstream control segments. However, there was a high incidence of animals having positive scores in the villi of control tissue.

The number of β-Gal-positive cells observed in the experimental and control segments of the above rats are presented in Table 1B. In the crypts, where background staining was not seen in control tissue, experimental segments displayed 2–11 positive epithelial cells in the four sections examined. Figure 1 shows an example of the histology. Although sectioning was performed transverse to the long axis of the intestine, crypts and villi were cut transversely at times. The upstream control section in Fig. 1A shows crypts in complete cross section, whereas the experimental section (Fig. 1B) is somewhat at an angle. Note that control tissue shows no blue reaction product whereas the experimental section (*i.e.*, the ligated region that was exposed to the vector) has several cells with a distinct positive reaction in the cytoplasm (see arrow). Because of the angle of the experimental section, positive cells were seen at three different levels of the crypts: The three crypts in the uppermost part of the picture (solid vertical arrows) are sectioned somewhere near their middle, the crypt in the center of the picture (open arrow) is sectioned close to the base (thus, the more dense appearance of the epithelial cells), and the two lowest crypts (horizontal arrow) are sectioned right at the base (thus showing no lumen).

In contrast to the clear results in the crypts, background β-Gal staining on the villi was a serious problem. As can be seen in Table 1B, the number of positive villus cells was just as high in upstream control tissue as in experimental tissue. Sham-operated animals (data not shown) exposed to culture medium instead of the retroviral vector showed a similar number of positive cells as in the villi of upstream segments. Because of this high incidence of endogenous β-Gal in villus epithelium, an alternative animal model/reporter gene system was sought.

TABLE 1. EXPRESSION OF CYTOPLASMIC β-GAL IN RAT TISSUES

			Crypt epithelium	*Villus epithelium*
A. Proportion f animals positive[a]				
Experimental segment			3/3	2/3
Control segment			0/3	3/3
		Number of sections examined	*Total number of positive cells*	*Total number of positive cells*
B. Number of positive cells				
Experimental segment:	Rat #2	4	2	2
	Rat #4	4	5	2
	Rat #9	4	11	0
Control segment:	Rat #2	4	0	2
	Rat #4	4	0	1
	Rat #9	2	0	3

[a]Data are shown as the number of animals with one or more positive scores in any of the sections taken from either the experimental segment or the upstream control segment.

β-*Glucuronidase expression in MPS VII mice*

Table 2A shows that experimental segments of 2 out of 5 mice exposed to the β-glucuronidase vector showed positive epithelial cells in both villus and crypt regions. There were no positive cells in the upstream control tissue of vector-treated mice or in any segments from sham-treated animals. Table 2B shows the number of positive cells observed in both crypt and villus epithelium. It can be seen that the incidence of positive epithelial cells is substantially higher on the villi than in the crypts. Of interest is the fact that in one of these mice (#24), positive villus cells were all localized on two to three adjacent villi. Examples of histology from mouse #24 are shown in Fig. 2. Figure 2A shows that upstream control tissue was devoid of the pink reaction product of the β-glucuronidase assay. In contrast, the tissue from the experimental segment displayed substantial pink staining that could be followed through 15 adjacent sections. Figure 2B, C, and D show examples at different intervals throughout this region. These sections being 8 μm thick, it can be concluded that cells from that region were expressing the β-glucuronidase gene over at least a 120-μm-long segment. None of the sham-operated mice exposed to the culture medium instead of the retroviral vector showed any positive cells.

DISCUSSION

The experiments described in this paper represent an *in vivo* attempt t explore the feasibility of using the intestinal epithe-

lium as a site for somatic gene therapy. Given the continual proliferation of this tissue, retroviral vectors were the most appropriate choice because retroviruses are known to infect dividing cells and to integrate their genome into the host DNA. Moreover, we had previously established that the mRNA for the ecotropic retroviral receptor is expressed in the intestinal mucosa and appears to be more abundant in proliferating epithelial cells than in differentiated cells (Puppi and Henning, 1995). We had also conducted studies with IEC-6 cells, a rat small intestinal crypt cell line, and found that these cells are readily transduceable by ecotropic retroviral vectors (Noel *et al.*, 1994).

The initial animal model we chose to explore was the Sprague-Dawley rat. Given the complex morphology of the intestinal mucosa, the use of a reporter gene that could be detected histochemically was preferred. Thus, in the rat experiments, we used the Zen$^+$β-gal retroviral vector, which has bacterial β-Gal as the reporter gene. All slides were scored blindly by two independent observers. Using this approach, appreciable numbers of false positives were found on the villi in both upstream control tissue and tissue from sham-operated animals. Because the latter animals had not been exposed to the retroviral vector, we conclude that this background staining represents endogenous β-Gal activity, most probably lactase *en route* to the apical membrane. The fact that these background problems were confined to villus epithelial cells (as compared with crypt epithelial cells) supports this conclusion, because lactase is known to be expressed specifically on the villus (Dudley *et al.*,1992; Duluc *et al.*, 1993). Although no conclusions regarding expression of the reporter gene can be drawn from the villus data, the crypt data are not compromised. In crypt epithelial cells, the consistent finding of β-Gal-positive cells in tissue exposed t the retroviral vector suggests that successful gene transfer had indeed occurred.

To obviate the villus background problems in our rat/β-Gal studies, we sought an alternative model in which the reporter gene could still be detected histochemically but in which the background in nonexposed segments would be zero. The use of β-glucuronidase as a reporter gene in MPS VII mice appeared to fulfill both of these criteria. In agreement with earlier studies of other tissues (Birkenmeier *et al.*, 1991), we found the intestinal epithelium of adult MPS VII mice to be lacking β-glucuronidase as assessed by histochemistry. Just as with our β-Gal studies, all slides were scored in a blinded fashion. Both sham-operated and upstream control tissue showed no β-glucuronidase staining. In contrast, experimental segments showed clear evidence of reporter gene expression in both the crypt and villus epithelium. Although luminal contents (*e.g.*, nutrients, bacteria, milk) might contain β-glucuronidase activity, the present data, in which a total of 1,098 sections of control tissue (upstream and sham-treated) yielded no positive intestinal epithelial cells, indicate that the positive cells in the treated animals were expressing β-glucuronidase activity from the vector.

Within the intestinal crypts, there are two potential populations of target cells for retroviral vectors, namely the stem cells and the transit population of dividing cells. Given that cells take approximately 36 hr to pass through the transit zone (Potten and Loeffler, 1987), the persistence of the reporter gene in the crypts 6 days and 4 days following gene transfer in the rats and mice, respectively, suggests that stem cells were transduced. However, definitive proof for this will require studies at longer time periods.

We conclude from both the rat and mouse studies that retroviral transduction of the intestinal epithelium is possible. With existing techniques, however, the efficiency of transduction is very low. For example, our best rat showed an average of approximately three positive crypt cells per intestinal cross section. Given that these sections contained an average of 40 crypts and that each crypt averaged 50 cells in length, there were approximately 2,000 crypt cells per section. Thus, the incidence of positive crypt cells in the rats was in the order of 3/2,000 or 0.15%. Similar calculations for our best mouse gives approximately the same incidence. Moreover, in the mouse studies, only 40% of experimental mice displayed transduction. Similar degrees of variability have been observed in other tissues fol-

TABLE 2A. NUMBER OF MPS VII MICE EXPRESSING β-GLUCURONIDASE IN INTESTINAL EPITHELIUM

Group	*Segment*	*Crypt*[a]	*Villus*[a]
Vector-treated	Experimental	2/5	2/5
	control	0/5	0/5
Sham-treated	Experimental	0/4	0/4
	control	0/4	0/4

[a]Data shown as the number of animals with positive crypt or villus epithelial cells compared with the total number of animals in that group.

TABLE 2B. NUMBER OF β-GLUCURONIDASE-POSITIVE CELLS OBSERVED IN INTESTINAL EPITHELIUM OF MPS VII MICE

Identification of tissues	*Mouse*	*Number of sections examined*	*Total number of positive cells*	
			Crypt	*Villus*
A. Vector-treated mice[b]				
Experimental segment:	#15	47	2	9
	#24	72	5	80
Control segment:		486	0	0
B. Sham-treated mice[b]				
Experimental segment:		293	0	0
Control segment:		319	0	0

[b]Data for positive tissue are shown for individual mice; those for control tissue are shown collectively.

FIG. 1. Histochemical assays for β-Gal in crypt region of upstream control tissue (A) and experimental tissue (B). Experimental tissue had been exposed to the Zen$^+$β-gal retroviral vector 6 days prior to sacrifice. The two sections shown were taken from the same animal. Magnification, 400×.

lowing exposure to retroviral vectors (Hatzoglou *et al.*, 1990; Clapp *et al.*, 1991; Kay *et al.*, 1992). Generic causes of such variability include lability of the vectors and the relatively small proportion of the cell cycle during which exposure must occur (Miller *et al.*, 1990). In our experiments, other contributors to the variability and low efficiency of gene transfer could have been inadequate numbers of vector particles and poor penetration of intestinal crypts. Although experimental segments were distended to open the crypts as described by Harris *et al.* (1988), in these experiments there was no attempt to remove mucus. Subsequent studies in our laboratory have established conditions for *in vivo* mucus removal (Sandberg *et al.*, 1994) that should be employed in future studies of gene transfer into this tissue.

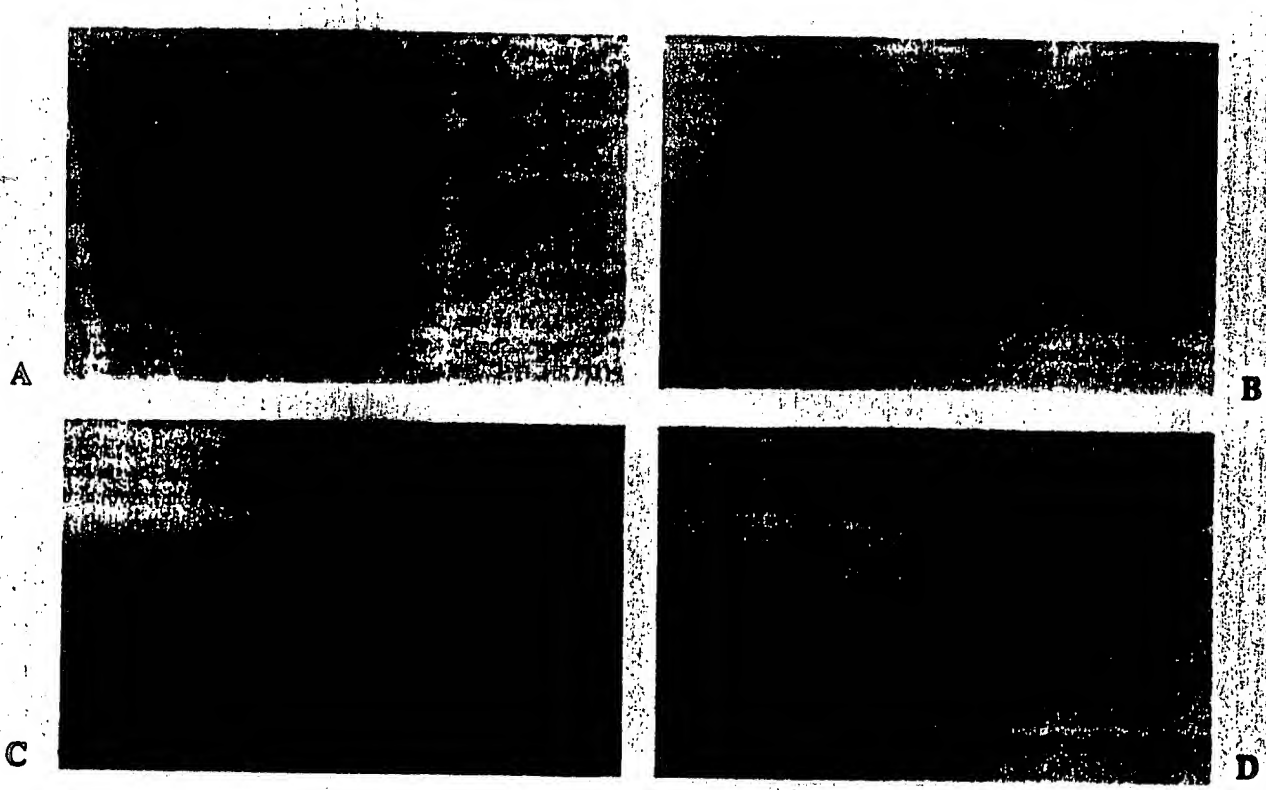

FIG. 2. Histochemical assay for β-glucuronidase in upstream control (A) and experimental (B, C, D) tissues from an MPS VII mouse. Experimental tissue was exposed to the NTK-βGEO retroviral vector 4 days before sacrifice. The sections shown were taken from the same animal (#24). Magnification, (A) 100×; (B, C, D) 200×.

In summary, we have demonstrated the feasibility of *in vivo* gene transfer into the intestinal epithelium using retroviral vectors. Clearly, there are problems to be overcome to achieve high-efficiency gene transfer as well as to decrease the variability between animals. Nevertheless, the potential advantages of the intestine as a site of somatic gene therapy indicate that further studies with this system should constitute a promising avenue of future research. With a view to ultimate clinical application, it is important to keep in mind that, although our studies with rodents used a surgical approach, in the human, luminal delivery of vectors to both the small and large intestine is conceivable *via* upper and lower endoscopic procedures, respectively. This is an approach that we are presently investigating in the large intestine of rats. Even if efficiencies of transduction remain relatively low, the ease of endoscopic delivery would allow multiple deliveries of vector to be performed, if necessary. Likewise, if the stem cell population proves difficult to transduce, repeated deliveries aimed at the transit cell population may also have therapeutic value.

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